Deactivation of oospores of *Phytophthora* taxon Agathis

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EXECUTIVE SUMMARY

Objective

The objectives of this work were to determine the efficacy of a variety of treatments to deactivate oospores of PTA:

- Determine if high concentrations of Trigene will kill ooospores
- Determine if exposure to seawater (seawater equivalent) for periods of 1 or 15 minutes will kill oospores
- Determine the effect of the liquid fumigant metam sodium on oospores in soil
- Determine effect of high and low pH levels on oospore viability
- Determine the time/temperature combinations required to kill oospores in solution
- Determine the time/temperature combinations required to kill oospores in soil

Key Results

Sensitivity of PTA oospores to Trigene
Trigene was tested at a range of concentrations (2%, 5%, 10% product) using two exposure times (30 min and 120 min). None of these Trigene treatments had any significant effect on the percentage of positively stained oospores compared with a deionised water control.

Salt water immersion
A short immersion in salt water had a negligible effect on PTA oospore survival.

Fumigation with metam sodium
Fumigation of soil and a soil/sand mix with metam sodium applied at three rates did not reduce the percentage oospores staining positive in either soil type or at any of the rates tested. Fumigated samples actually had significantly higher counts than untreated samples in soil.

Immersion in solutions of differing pH
The effect of eight solutions with pH ranging (increments of one) from 3 to 10 and with exposure times of 2, 4, 12, 24 and 48 hours on oospore survival were tested. At the lowest tested level (pH=3) the percentage of positively stained oospores was moderately reduced to 14% and did not differ significantly with exposure time. Although there was also a reduction in staining at higher pH levels, this only occurred for extended exposure times. At pH levels 9 and 10 the 24 and 48 hour treatments reduced viability to levels below all other treatments. There were no viable spores after 48 hours exposure to pH 9 or pH 10. The optimum pH for oospore viability tended to be in the middle ranges (pH 6-8).

Heat treatments
Exposure to temperatures of 55°C for 4 hours in solution was effective at reducing viability to extremely low levels. Shorter exposure times were applied with increasing temperature (e.g., 0.5 hours at 70°C), and all time/temperature combinations were equally effective. Oospores embedded on micromesh and buried in soil were almost as responsive to temperature as those in heated solution with a 4 hour treatment at 60°C and 70°C giving mean stained spores of only 3-6%. Wet soil gave significantly better results than dry soil though the differences were small. Occasional stained spores were observed in some replicates of many treatments though it is possible that these occasional stained spores were more of an artefact than a reality. Exposing oospores embedded on micro-mesh to a dry heat of 70°C for 4 hours gave a reduction in viability of only 30%, emphasising the importance of moisture in achieving the desired result.

Conclusions

For practical purposes the application of heat is likely to be the most effective and practical treatment for deactivation of PTA oospores in samples of contaminated soil collected from equipment or footwear. Results of this work indicate that temperatures of 60 - 70°C applied to wet soil or through a steam applicator for periods of 4 hours would result in total kill. A safety margin could be applied. At temperatures higher than 70°C shorter treatment periods are likely to be effective.

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Introduction

Phytophthora taxon Agathis (PTA) was first reported from a stand of unhealthy kauri at Kaitoke Creek, near Whangaparapara on Great Barrier Island (Gadgil 1974) and was recorded then as *Phytophthora heveae*. Subsequent molecular studies have indicated that it is likely to be an undescribed species of *Phytophthora*. Symptoms of disease in the Kaitoke Creek stand included yellowing of foliage, canopy thinning and lesions on the lower trunk which sometimes encircled the stem and were bleeding copious amounts of resin (kauri gum). Lesions were also found on the main roots. Some trees had died. PTA was recovered from the stem lesions, roots and the soil. It was also found in soil in a second stand of kauri on Great Barrier Island at Kaiaraara where the trees had healthy crowns and no sign of gummosis on the stems. In pathogenicity tests PTA has been demonstrated to be capable of rapidly killing kauri seedlings (Gadgil 1974; Beever et al. 2010).

PTA has subsequently been isolated from soil in a number of locations in Northland and Auckland where there are unhealthy kauri (Waipara et al. 2010; S. Bellgard pers. comm.; A Beauchamp pers. comm.). The distribution of PTA has not however been systematically established. Beever et al. (2009) proposed that collar rot caused by PTA is an emerging disease caused by an introduced pathogen that is spreading slowly from a number of disease foci. It therefore poses a threat to kauri, both at the individual and at the population level, with flow-on effects to kauri ecosystems.

Understanding of the epidemiology of the disease is limited. We know little about the length of time for a visible lesion to develop after infection has taken place; the influence of site factors on survival of PTA in soil or on initiation of infection. We don't know if other species of *Phytophthora* (five species of *Phytophthora* have been recorded from soil in kauri forests) also play a role in kauri collar rot. However, to-date, the only *Phytophthora* recovered from symptomatic kauri trees is PTA.

The goal of the Kauri Dieback Joint Agency Response (KDJAR) is to slow the spread of PTA and to undertake measures to limit its effect on individual trees and on ecosystems. An important part of this process is to ensure that there is no viable inoculum of PTA in any material before it enters or is removed from kauri field sites. Material could include bark and wood, or mud and soil washed from footwear, equipment and vehicles used in track maintenance etc. Propagules of PTA could include mycelium, sporangia, zoospores and oospores. Although the first three of these propagule types are readily killed by exposure to fairly low levels of heat or by chemicals such as disinfectants and biocides the deactivation of oospores presents more of a challenge.

Oospores are the most persistent of the propagules types produced by *Phytophthora* spp. and the main survival propagule in soil for many species, especially those that do not produce chlamydospores (Erwin & Ribeiro 1996). When conditions become favourable, oospores germinate and develop mycelia or produce sporangia. Typically oospores will survive environmental conditions that are lethal to mycelia and sporangia. For example the mycelium of *P. kernoviae* ceases to grow at ~26°C and is killed after several hours at 35°C but oospores require temperatures from 50 - 60°C for periods of several hours (Widmer 2011; Arhipova et al. 2012, unpublished data) before all are killed. Germination of oospores is influenced by oospore age, nutrition, temperature, light, chemical treatments, and probably by further unrecognised factors. Oospores are usually formed in diseased plant tissue, both on infected plants and in fragments of decaying tissue on the ground and in soil (Erwin & Ribeiro 1996). They may also be found free in soil after degradation of infected plant tissue. It has also been shown experimentally that sporangia and mycelium of *P. kernoviae* may survive in soil by forming oospores (Widmer 2011).

Once oospores are mature they may remain viable but in a dormant state for several years. Conditions that break dormancy and promote germination at any one time may not lead to the germination of all of the spores. Hence determination of whether material such as plant tissue or soil contains viable inoculum requires methods other than plating onto agar medium and evaluating colony formation. The ability of those oospores characterised as alive but dormant to survive and germinate at a later stage remains an issue for the development of mitigating procedures when managing *Phytophthora* diseases. At low-moderate temperatures dormant spores in soil have been demonstrated experimentally to germinate after many months albeit at low percentages (Widmer 2010; 2011).

Inactivation of oospores

A variety of techniques have been used to kill *Phytophthora* infected plant material and soil so that it no longer presents a risk of contaminating new material or environments. Heat treatment, in a range of application methods, has been the most frequently employed. Other techniques include disinfecting chemicals and fumigants. Much of the literature reporting on the elimination of *Phytophthora* from various substrates deals with mycelium and the asexual spore stages and there is comparatively little information on treatments to kill oospores.

In this work we have been asked to look at the effect of the disinfectant Trigene, salt water immersion, soil fumigation with metam sodium, pH solutions and heat treatments applied for different time periods on PTA oospore viability. Some background on these treatments follows.

Trigene

The active ingredients of TriGene™ (II) Advance (TriGene) are a group of halogenated tertiary amines. The primary components are polymeric biguanide hydrochloride, alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride. TriGene™ (II) Advance contains 12% active ingredient. The product information states that it is effective against a range of micro-organisms including bacteria, viruses, and fungi at its recommended label rate of 2% product. Oomycetes are not mentioned but the product is widely used for the cleaning of footwear and equipment that have been contaminated with soil with the deactivation of *Phytophthora* spp. as the primary target. The effectiveness of Trigene (and several other disinfecting chemicals) for treating life stages of PTA, including oospores, has been extensively tested by Bellgard et al. (2009) who tested oospore viability using the tetrazolum bromide vital stain. Although no active (red or purple) oospores were found after treatment a high percentage (slightly greater than the combined red/purple plus pink oospores of the untreated control) of the oospores were pink and therefore presumed dormant and (at least some of them) capable of germinating at a later stage. The efficacy of higher concentrations of Trigene on oospore viability is unknown, hence the request to test 5% and 10% solutions of product.

Salt water

The salinity of water in the world's oceans ranges from 3.1 to 3.8% with an average of about 3.5% (35 g/L, or 599 nM. Seawater salinity is made up of a number of ions though Na+ and Cl-, at concentrations of ca. 0.469 and 0.546 mol/kg respectively, are the major components. Mg++ at 0.05 mol/kg is the next most abundant with Ca++, SO4 and other ions in much lower quantities. The pH is in the range 7.5 to 8.4. It was considered that dissolving sea salt in deionised water would provide a solution that would bear sufficient resemblance to sea water for experimental purposes.

Fumigation

Soil fumigants (e.g. methyl bromide, chloropicrin, metam sodium) have been extensively used with considerable success to kill a wide range of soil pathogens though use of some such chemicals in this manner is not permitted under current regulations. Highly volatile fumigants must be injected into the soil and covered for a period of time. Metam sodium however is applied in liquid form as a drench which then releases methyl isothiocyanate.

Metam sodium has been used in the USA to successfully eliminate *P. ramorum* from potting medium (Lindeman & Davis 2008) and as a component of a programme in Australia to prevent spread of *P. cinnamomi* (Dunstan et al. 2010) at field sites with sandy soils. However neither *P. cinnamomi* nor *P. ramorum* form oospores in the natural environments where these trials have been carried and the effect on these propagules of *Phytophthora* is not known. Lindeman & Davis (2008) included *Pythium irregulare*, which does form oospores, in their programme and reported the successful elimination of this species (determined by baiting with plant tissue) at the highest of the three rates that they tested (1ml/L potting medium).

Metam sodium (as Fumasol® Elliot Technologies) is available for use in New Zealand by registered operators. The recommended rate for seed bed application is 67 – 87 ml /m^2.

Acid/alkaline treatments

Many *Phytophthora* species have been shown to prefer acid soils over alkaline. A number of studies of tree/plant declines have investigated how the extent of root infection alters with changing pH values. This tends to be highly variable and is host/pathogen/drainage and soil type dependent. As zoospores are the primary infective propagules the effect of pH on infectivity is likely related to the effect on zoospore production, activity and encystment.

Heat treatments

The optimal temperature for growth of PTA colonies is 26°C with growth ceasing at 29°C (I. Horner pers. comm., 6 isolates tested). Experimental work with other species of *Phytophthora* has shown that mycelium will survive temperatures considerably higher than that at which growth ceases. For example mycelia of both *P. ramorum* and *P. kernoviae* (growth ceases at 27°C and 26°C respectively) were very resilient to dry heat treatment, with isolates from both species surviving a 15, but not a 30 minute, treatment at 60°C. By extending the treatment time to 60 minutes for *P. ramorum* and 120 minutes for *P. kernoviae* it was possible to kill mycelium at lower temperatures. Wet heat treatments also made it possible to reduce the time required to kill mycelia. At 40°C mycelia were killed after 15 minutes and at 37.5°C after 90 minutes (Turner et al. 2008). The resting structures, oospores and chlamydospores, required considerably higher levels of heat to ensure they were killed (Widmer 2012). PTA does not form chlamydospores but readily produces oospores.

Heat can be applied to environmental samples through the use of steam, dry heat in ovens/heat units or naturally through composting. Although the temperature at which mycelial growth of PTA ceases is known the temperature/time combination required to kill PTA mycelium, sporangia or oospores has not been determined and it is essential to know these figures for any heat-based treatment option of soil to be evaluated. Based on information on other *Phytophthora* species a percentage of oospores would be expected to survive a short period (eg 1-several hours) at ~60°C (wet heat) with a longer period ensuring that all oospores are killed.

Evaluating oospore viability

Several techniques are used to evaluate the viability of oospores. The vital stain tetrazolium bromide is frequently employed to test oospores in solution and the oospores are characterised as dead (unstained or black), alive but dormant (pink) or active (red or purple) (e.g. Widmer, 2010, 2011; Etxeberria et al., 2011) (Fig. 1). This is often complemented with examination of suspensions under a high-powered microscope to check for germination of the oospores. There is correlation between germination percentages and counts of living (pink and purple/red) spores from the vital staining though the germination per cent is lower than that achieved using tetrazolium bromide staining. This result is consistent with the expectation that not all dormant spores will germinate even if conditions are favourable. Other techniques to visually test viability include the use of salt solutions which cause plasmolysis of viable spores while non-viable spores are not affected (Jiang & Erwin 1990; Sutherland & Cohen 1983).

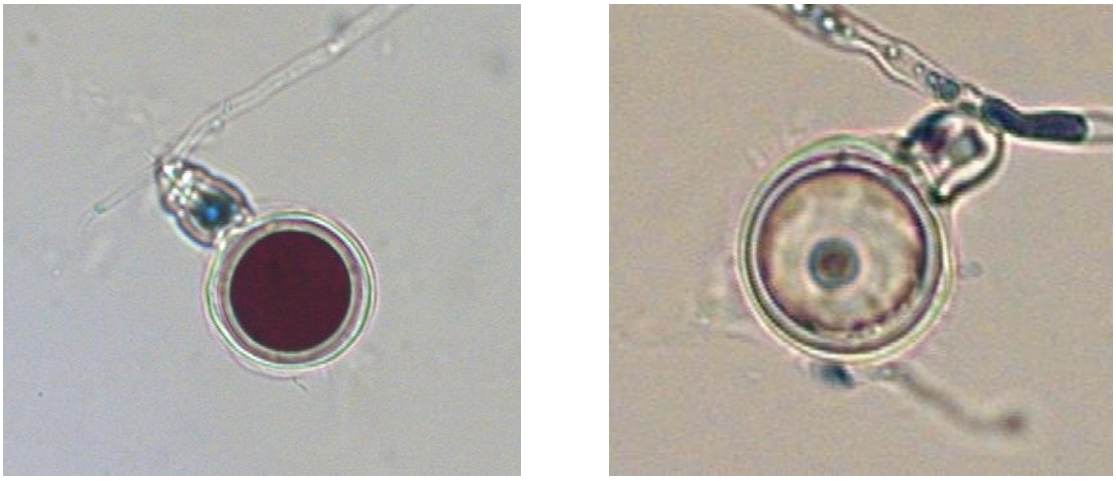

Fig. 1: Purple stained (a) and unstained (b) oospores following tetrazolium bromide treatment

Objectives

The objectives of this work were to determine the efficacy of a variety of treatments to deactivate oospores of PTA:

- Determine if high concentrations of Trigene will kill ooospores
- Determine if exposure to seawater (seawater equivalent) for periods of 1 or 15 minutes will kill oospores
- Determine the effect of the liquid fumigant metam sodium on oospores in soil
- Determine effect of high and low pH levels on oospore viability
- Determine the time/temperature combinations required to kill oospores in solution
- Determine the time/temperature combinations required to kill oospores in soil

Materials and Methods

PTA isolates

Isolates used in the preparation of oospore suspensions were obtained from the Forest Research Culture Collection (NZFS), Rotorua, New Zealand. Three isolates were used, all from soil samples collected in Waipoua Forest in 2011. These were:
NZFS 3681, 3687 and 3709

Formation of oogonia

Small blocks (c. 1 cm × 0.5 – 1 cm) of agar plus mycelium were cut aseptically from colonies growing on carrot agar[1] in Petri plates and immersed in clarified, sterile V8 broth[2] in plastic Petri dishes. Plates were sealed with Parafilm™ ribbon and held at 20 °C in the dark to allow oospores to form on the new mycelium growing into the broth.

[1] 200 g washed and diced carrot pieces blended in 500 mL deionised water, 15 g agar added, made up to 1 L with deionised water, autoclaved at 121 °C for 15 min and agitated during pouring to disperse carrot fragments

[2] 100 mL V8 juice (Campbell's Soups Australia™) and 4g $CaCO_3$ (powdered chalk) mixed with 100 mL deionised water, vacuum filtered 3× through 1 layer of Whatman No. 1 filter paper and 2× through 2 layers, made up to 1 L with deionised water and autoclaved at 121 °C for 15 min.

Fig. 2: Tube drive used to separate oospores from mycelium

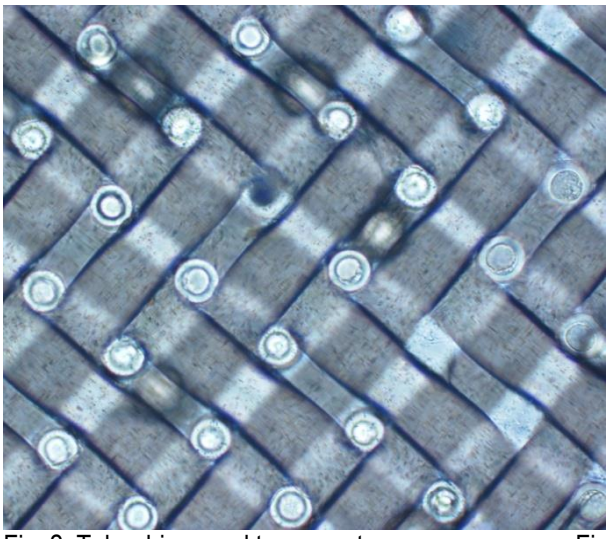

Fig. 3: Oospores of PTA embedded on micromesh

At 6-8 weeks the new mycelial growth was then cut away from the agar blocks and suspended in deionised water. This solution was then placed in an ULTRA-TURRAX® Tube Drive (Fig. 2) for 20-30 seconds at maximum power which separated the oospores from the mycelium without disruption. The resultant oospore suspension was then used directly or oospores were embedded in nylon net filter discs. Discs were 47 mm diameter, 20 µm mesh (NY2004700 Millipore™) and 5mL aliquots of oospore suspension were filtered through under suction leaving the majority of oospores trapped in the pores (Fig. 3). Once prepared the pieces of mesh were allowed to dry and stored at 4°C in the dark.

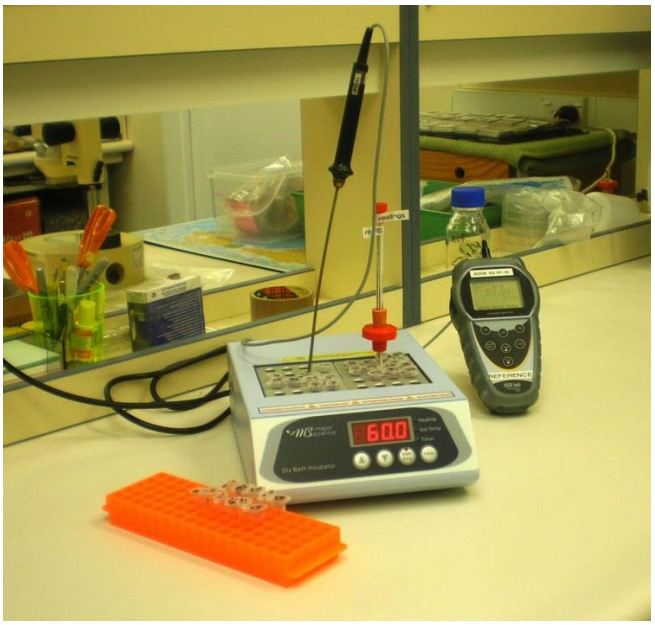

Fig. 4: Micro-centrifuge tubes containing oospores under treatment in the heat block

Vital staining with tetrazolium bromide

After treatment the oospore solution or pieces of micro mesh were placed in individual 2ml micro-centrifuge tubes; these were kept at 4°C for 4 -14 days before staining. This delay was to ensure that any spores slow to respond to the treatment would have died before the microscopic evaluation took place. A 0.05% solution of tetrazolium bromide (1.5ml) was added to each tube. Tubes were then placed in a heat block (Fig. 4) at 35°C for 48 hours. Pieces of mesh were then extracted from the solution, placed on a microscope slide and examined at 200x magnification. Oospores were assessed as pink, red/purple, unstained or 'other' (included spores that were black, blue or empty) (Figs. 5 and 6).

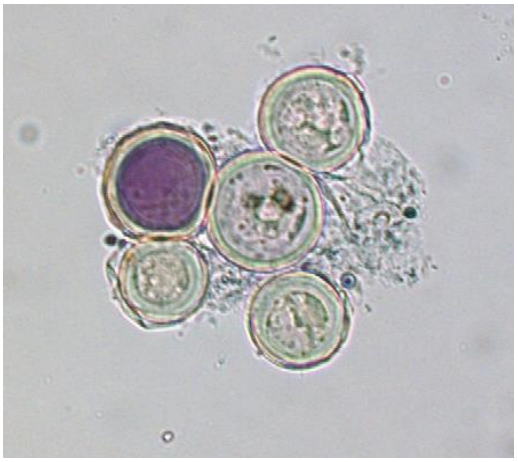

Fig. 5: One positively stained and several unstained oospores after tetrazolium bromide treatment

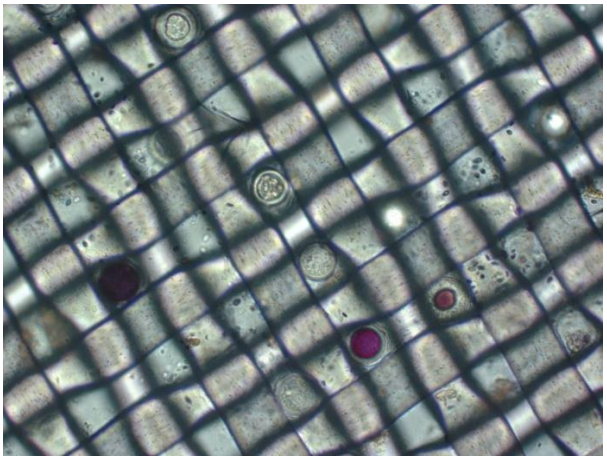

Fig. 6: Stained and unstained oospores on micromesh

Trigene

Product solutions of 2% (0.24 % a.i.), 5% (0.6 % a.i.), and 10% (1.2 % a.i.) were prepared. Pieces of micro mesh with oospores of isolates NZFS 3687 and 3709 embedded were immersed in solution for periods of 30 and 120 minutes. Three replicates for each treatment were applied and the entire experiment was repeated once. Equivalent controls were carried out using sterile deionised water. The pieces of mesh were held for six days before addition of the tetrazolium stain and heating.

Salt water immersion

Pieces of micro mesh with oospores of isolates NZFS 3681 and 3709 embedded were immersed in 3.1% and 3.8% solutions of sea salt for periods of 1 and 15 minutes. Three replicates for each treatment were applied and the entire experiment was repeated once. To satisfy curiosity (that the prepared solution would give the same results as natural seawater) and for an additional comparison seawater collected from Tauranga harbour was also tested. Equivalent controls were carried out using sterile deionised water. After treatment the pieces of micro mesh were placed in individual 2ml micro-centrifuge tubes and held at 4°C for 10 days before addition of 0.1% tetrazolium bromide solution and heating for 48 hours.

Fumigation

A light soil (collected from a Scion Nursery bed) and a 70/30 sand/soil mix were tested. The soil and sand/soil mix were moistened and mixed well. Twenty four plastic zip-lock bags were filled with 500 gm of soil or the sand/soil mix; 12 bags of each. Bags were packed into trays and arranged so that there was a consistent surface area of ca. 40 cm2. Three concentrations of metam sodium in deionised water were prepared (25, 12.5 and 2.5 ml /L). A 20 ml application to the surface of the soil would therefore give an equivalent rate of 125, 62.5 and 12.5 ml /m2. Three replicate bags for each metam sodium concentration /soil type combination were prepared. Control treatments were carried out with deionised water. Pieces of micro mesh with oospores of isolates NZFS 3687 embedded were buried in each bag to a depth of ca. 4 cm prior to application of the chemical. The bags were sealed and incubated in a fume hood for 48 hours at room temperature after which the bags were opened to allow the methyl isothiocyanate to vent. Open bags remained in the fume hood for a further 5 days before the pieces of micromesh were removed and treated with tetrazolium bromide. Three pieces of mesh with embedded oospores that had been held in the laboratory at 4°C during the fumigation treatment period were included in the staining procedure as laboratory controls.

pH treatments

Eight solutions with pH ranging (increments of one) from 3 to 10 were prepared using deionised water adjusted with hydrochloric acid (HCl) or sodium hydroxide (NAOH). Controls were deemed to be the middle range pH levels. Pieces of micro mesh with oospores of isolate NZFS 3681 and 3709 embedded were immersed in each solution for periods of 2, 4, 12, 24 and 48 hours. . After treatment the pieces of micro mesh were placed in individual 2ml micro-centrifuge tubes and held at for 12 days before addition of 0.1% tetrazolium bromide solution and heating for 48 hours.

Heat treatments

Stage 1 – oospores in solution:

Three temperatures (55, 60 and 70^{0}C) were tested with the time regimes decreasing with increasing temperature (Table 1). Oospore suspensions of three isolates (NZFS 3709, 3681 and 3687) were prepared as described. For each treatment (time/temperature/isolate combination) one ml of oospore suspension was placed in each of three 2ml micro-centrifuge tubes. These were placed in the heat block which had been and exposure time measured from the time that the desired temperature was reached. This was determined by both a thermometer and the insertion of a temperature probe in a test vial (Fig. 1). After treatment was completed vials were removed from the block, held for four days before addition of 1 ml of 0.1% tetrazolium bromide solution and heating for 48 hours. Small aliquots of solution were placed on microscope slides and scanned for oospores.

Table 1 – Treatment temperatures and time periods for PTA oospores in solution

Temperature (^{o}C)	Exposure time (hours)
room temp	24
55	4, 6, 12, 24
60	4, 6, 9, 19
70	0.5, 1, 2

Stage 2 – oospores in soil - Trial 1:

Oospores of isolates 3681 and 3687 deposited on micromesh were used in the first round of treatments in soil. Pieces of mesh were buried in plastic pottles (100 mm diameter by 70 mm high) each holding 500 gm of nursery soil. The soil was slightly moist with moisture content averaging 37.75% (as determined by oven drying).

Fig. 7: Kilns in the Timber Drying Laboratory at Scion

Temperature treatments were carried out in the research kilns (Fig. 7) in the Timber Drying Laboratory at Scion. These kilns simulate a commercial timber drying kiln and have electric heating, variable speed fans and humidity control using a steam spray line and vents. Temperatures tested were 50, 60 and 70^{o}C for periods of 4 hours and 24 hours for all three temperatures, and also 1 hour for the 60 and 70^{o}C temperatures.
Three additional variables were tested:

- Containers of soil were covered tightly so that initial moisture content was maintained. No steam was supplied and the heat in the kiln was dry.
- Soil surface was exposed so that further soil drying occurred during the process. No steam was supplied and the heat in the kiln was dry.
- Soil surface was exposed and the steam sprayline was on most of the time so that RH was at 95-96%.

Fig. 8: Soil pottles for kiln treatments

A temperature probe inserted through the lid, or directly into the soil for uncovered containers to measure soil temperature (Fig. 8). Following a test run the rate of heat up was set to half an hour. Following treatment the micromesh pieces were removed from the soil, held for four days before addition of 1 ml of 0.05% tetrazolium bromide solution and heating for 48 hours. There were three replicates for each treatment.

Stage 2 – oospores in soil - Trial 2:

Pieces of micromesh with embedded oospores of isolate 3687 were buried in plastic pottles of nursery soil as for trial 1. Temperatures tested were 60 and 70^{o}C for periods of 1 and 4 hours. Two further variables were soil moisture; the soil was either slightly moist as in trial 1 or 100 ml of water was added to each container to give a soil that was wet. Containers were kept covered so that soil moisture content did not change during the treatment. A temperature probe was inserted through the lid. As a further test half-circles of the micromesh s were stapled to pieces of wood (Fig. 9) and placed in the kilns during the 70^{o}C 4 hour treatments. There were three replicates for each treatment.

Fig. 9: Exposed micromesh on wood and pottles containing soil for heat treatments

Kiln treatments are summarised in Table 2.

Table 2 – Treatments applied to PTA oospores in soil

Exposure method	Temperature (^{o}C)	Exposure time (hours)
Trial 1		
Mesh	room temp	0
Exposed soil/dry kiln	55	4, 24
	60	1, 4, 24
	70	1, 4, 24
Covered soil/dry kiln	55	4, 24
	60	1, 4, 24
	70	1, 4, 24
Exposed soil/steam applied	55	4, 24
	60	1, 4, 24
	70	1, 4, 24
Trial 2		
Mesh	room temp	0
	60	4
	70	1, 4,
Dry soil	60	1, 4,
	70	1, 4,
Wet soil	60	1, 4,
	70	1, 4,

Statistical analysis

Each of the treatments listed above was analysed as a separate trial using generalized linear mixed models. In all analyses, the dependent variable was the percentage of oospore staining positive, and this variable was assumed to follow an over-dispersed binomial distribution. The objective of each analysis was to test whether the experimental factors tested such as temperature, exposure time, or exposure method, significantly reduced the percentage of oospore staining. Experimental factors with p-values less than 0.05 were assumed to be statistically significant.. Multiple comparisons between treatment levels were conducted using Tukey's test. Isolate was included as an experimental factor in relevant trials, but results are not reported as treatment effects were similar for all isolates. Results are summarised using charts showing treatment means with error bars showing plus or minus one standard error (note however that the true mean can generally be assumed to lie within plus or minus 2 standard errors of the mean). All analyses were performed using the SAS (Version 9.2) GLIMMIX procedure.

Results and Discussion

Trigene disinfectant

There was no apparent difference between the two repeats of the trial and the results were combined for analysis. Neither trigene concentration (deionised water control, 2%, 5%, 10%) nor exposure time (30 min and 120 min) had any significant effect on the percentage of positively stained oospores (Fig. 10). Isolate NZFS 3709 had a consistently and statistically ($p<0.0001$) significantly higher percentage positively stained spores than NZFS 3687 but the neither of the two isolates responded to the Trigene treatments. Results for the two isolates have been combined in Fig. 10.

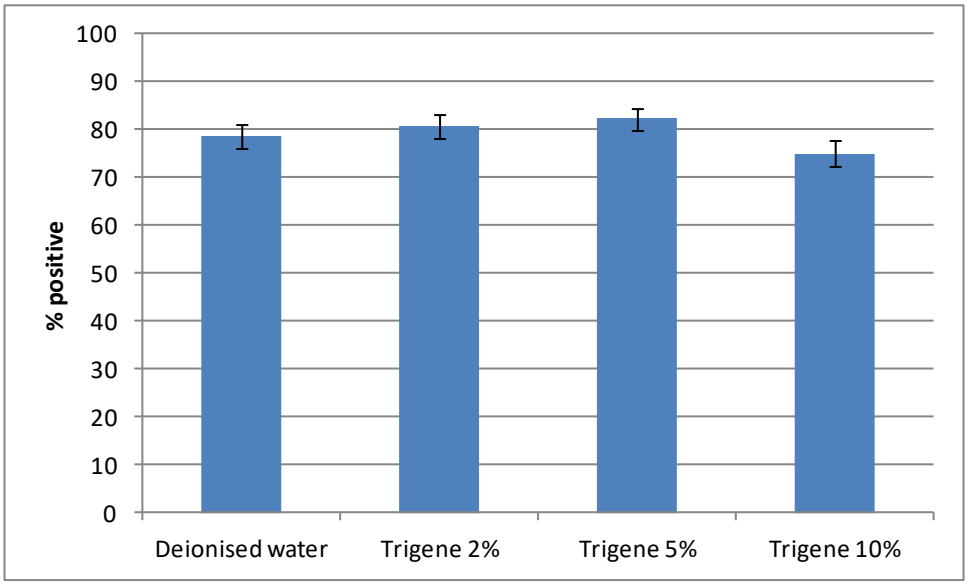

Fig. 10 - Mean % positively stained oospores for each treatment in the Trigene disinfection trial. Error bars show standard errors.

Trigene is biodegradable (Medichem International Ltd 2008) and has low mammalian toxicity. It is considered to have low environmental impact in comparison with some other chemical treatments which makes it a desirable product if this form of disinfection is required. Several trials examining the effectiveness of Trigene against PTA in which viability has been tested using direct plating of infected soil to Phytophthora-selective medium or through bioassay in which leaf-baits or other plant tissues are used in flooded soil samples have been carried out (Bellgard et al. 2009; Pau'uvale et al. 2012) In these trials there has been no colony formation of PTA, or other *Phytophthora* species on the selective agar media used. However Bellgard et al. (2009) found that after a 2% Trigene treatment although no active oospores (red/purple colour) remained a high percentage (slightly greater than the active plus pink oospores of the untreated control) were recorded. Our results parallel this finding.

Salt water immersion

There was a small reduction in percentage oospores staining positive for the two saltwater treatments compared with the control (Fig. 11). Although statistically significant, this reduction was inconsequential in practice. Exposure time (1 minute and 15 minutes) had no significant effect and results from these treatments have been combined for presentation. Results from the tests with water collected from Tauranga harbour matched those from the saltwater preparations (data not included).

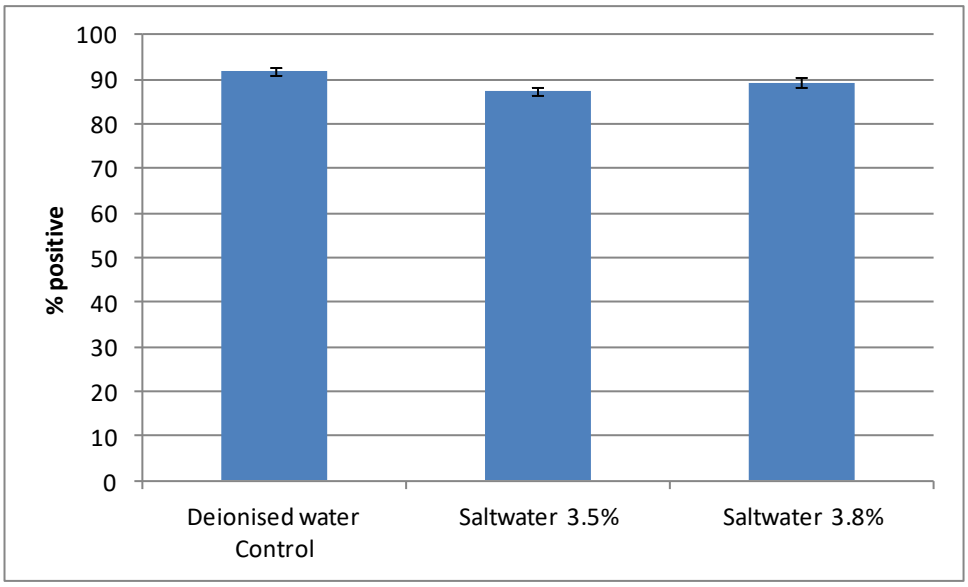

Fig. 11 - Mean % positively stained oospores for each treatment in the saltwater trial. Error bars show standard errors.

A number of researchers have looked at the effect of marine environments on mycelial growth of selected oomycetes and fungi. Duniway (1979) and Padgett (1984) both suggested that salinity levels have little or no effect on many *Phytophthora* spp. Wilkens & Field (1993) found that growth of two marine fungi was virtually identical in natural sea water and artificial sea water containing only the macro-constituents of sea water. In studying *Phytophthora polymorphica*, an estuarine species, they found it performed well in a wide range of salinity levels as measured by biomass production and also suggested that the genus Phytophthora is generally tolerant to such conditions.

A presentation by Rollins & Singer of Washington State University USA (presentation viewed on-line) tested the effect of a range of salinities on growth of P. ramorum for periods of 7 and 11 days. They found that mycelium growth rate decreased slightly with increasing salinity at these time periods.

Fumigation of soil with metam sodium

Fumigation of soil with metam sodium did not reduce the percentage oospores staining positive at any of the rates tested. In fact, fumigated samples actually had significantly higher counts than untreated samples (Fig. 12). There was no difference between the soil and the sand/soil mix and results were combined for analysis.

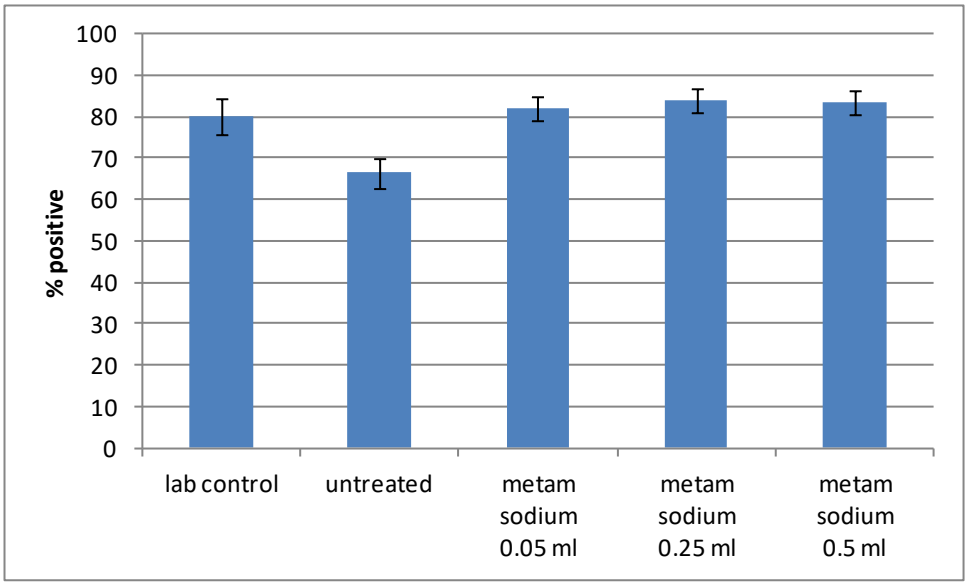

Fig. 12 - Mean % positively stained oospores for each treatment in the soil fumigation trial. Error bars show standard errors.

There was no difference between the 3 treatment rates of metam sodium and no difference between the two soil types. There was also no significant difference between any of these treatments and the laboratory controls. However the control treatments, in which only water was applied to the bags, had a significantly lower number of positively stained oospores than those treated with the chemical. This result is in contrast to those reported by other researchers, notwithstanding that oospores of *Phytophthora* spp. were not examined in the cited references.

Lindeman & Davis (2008) found that *P. ramorum* was eliminated at all rates of metam sodium tested. They included *Pythium irregulare*, which does form oospores, in their programme and successfully eliminated this species at the highest of the three rates that they tested (1ml/L potting medium).Dunstan et al. (2010) found that metam sodium was effective against *P. cinnamomi* at both of their study sites (sandy soils in which rainfall infiltration rate is high and surface water movement is low which limits progression of *P. cinnamomi* through the landscape) though they commented that they did not identify the life-stages of the pathogen at either site and the efficacy of the treatment against resting stages needed to be confirmed. Another fumigant, considered by some to be a safe alternative to methyl iodide and registered for use in New Zealand was reported by Ohr et al. (1996) to be effective in killing tested species of *Phytophthora* in soil.

In all the research studies cited the method of determining viability of inoculum was through direct plating or by bioassay. All of the *Phytophthora* spp. tested are hereothallic and it is likely that oospores were not present. The results obtained from vital staining of PTA oospores were somewhat surprising and if soil drench or fumigation treatments were thought worth pursuing then further experimental work to aid understanding of the interaction between fumigant and oospores would be required. There are however problems anticipated with the operational use of such treatments. These include:

- Application is highly regulated
- Must be applied by a registered operator
- Costly (e.g. relative to heat treatments)
- Treatment time may exceed 24 hours

pH treatments

There was a significant reduction in percentage oospores staining positive with reducing pH (Fig. 13). At the lowest tested level (pH=3) percent positive stain averaged 14% and did not differ significantly with exposure time (which varied from 2 to 48 hours). Although there was also a reduction in staining at higher pH levels, this only occurred for extended exposure times. At pH levels 9 and 10 the 24 and 48 hour treatments reduced viability to levels below all other treatments. There were no viable spores after 48 hours exposure to pH 9 or pH 10. Although there was some variation around the results, particularly an outlier at pH 6 for 2 hours, the general trends are consistent. The optimum pH tended to be in the middle ranges (pH 6-8) though there was some variation around this area. For the 2 hour treatment a pH level of 3 reduced viability to 18%, 22.3% and 21.9% respectively of that recorded for pH levels 6, 7 and 8.

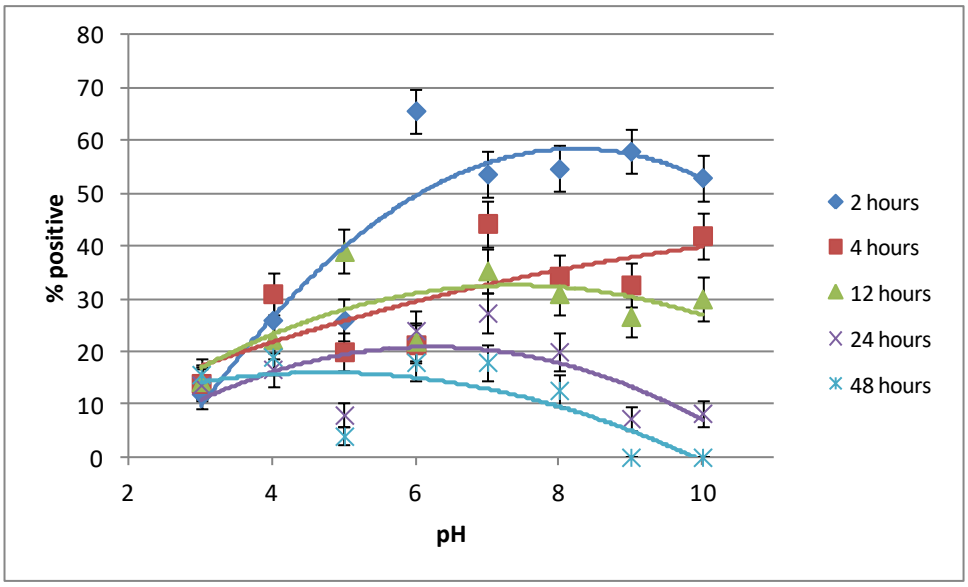

Fig. 13 - Mean % positively stained oospores for each treatment in the pH trial. Error bars show standard errors. Lines show quadratic regression curves fitted against pH for each exposure time.

The results from these tests contrasted from those reported by other researchers for the reaction of zoospores and sporangia to varying pH levels. Studies have focussed on the effect of pH on zoospore survival and as these spores lack cell walls and encysted spores or cysts have thin walls, they are presumably more vulnerable to pH stress than oospores or chlamydospores.

In soil systems *Phytophthora* species are considered tolerant to a wide range of pH from 3.8 to 9.0 (Weste 1983). Kong et al. (2009, 2011) tested the effect of pH on zoospore survival of seven *Phytophthora* species commonly found in natural waterways The pH levels ranged from 3 – 11 and the *Phytophthora* spp were exposed for up to 7 days. The optimal pH differed among species, ranging from 5 to 9 but with the majority (5 species) at pH 7. At pH 5 to 11, the recovery rate decreased sharply after 1-day exposure for five of the seven species. In contrast, no change occurred in the recovery of any species even after a 7-day exposure at pH 3. However Benson (1984) found that sporangial production of *P. cinnamomi* was inhibited at a pH of 3.7 and lower.

Heat treatments

Stage 1 – oospores in solution:

Heating in solution significantly reduced percent positive staining oospores compared with an unheated control at all temperature/time combinations tested (Fig. 14). There were no significant differences between the 3 temperatures (55, 60 and 70°C), and no differences between exposure times (which were varied depending on temperature). Significant isolate differences were observed with staining for NZFS 3689 being significantly higher than NZFS 3687 and NZFS 3681 intermediate. As heat treatments were equally effective on all isolates, the combined results across all isolates are shown in Fig. 14.. Numbers of stained oospores were very low for all heat treatments

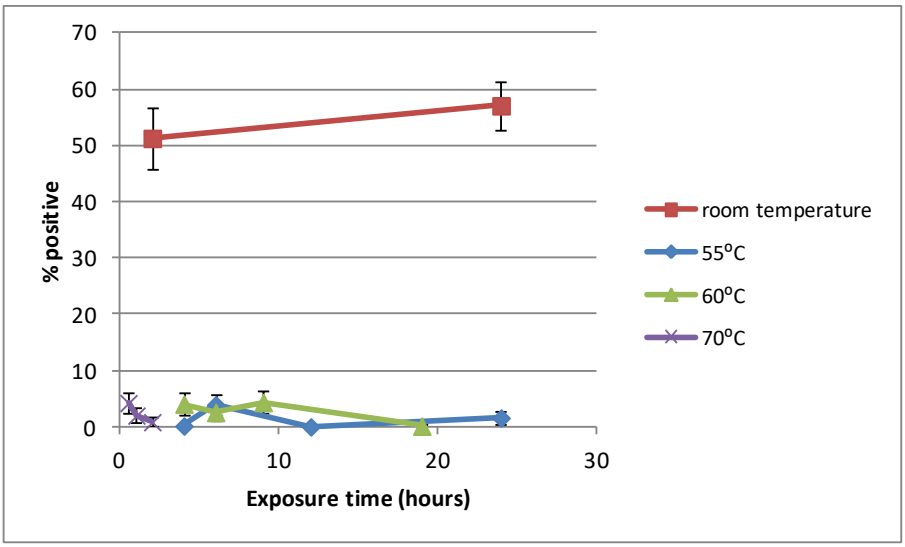

Fig. 14. - Mean % positively stained oospores by temperature and exposure time heated solutions. Error bars show standard errors

Stage 2 – oospores in soil

In Round 1 of this trial, all heat treatments were found to significantly reduce % positive stain compared with the two room temperature control treatments. Mean % positive staining of oospores in wet soil was marginally lower than in dry soil with no lid (p=0.038) with lidded dry soil intermediate. The two room temperature controls (exposed mesh and mesh in soil) did not differ significantly. Staining did not differ significantly with either the temperature of the heat treatment (50, 60 and 70°C) or exposure time (1, 4 and 24 hours) (Fig. 15).

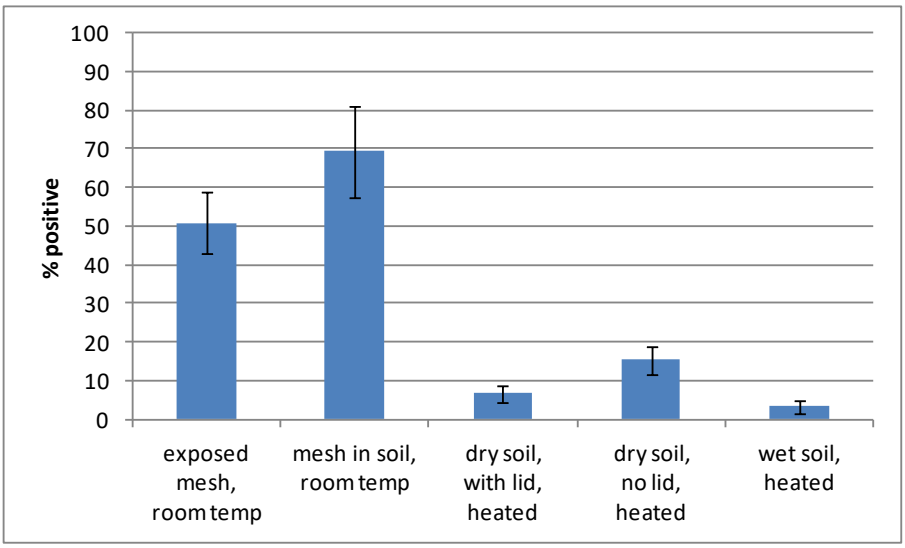

Fig. 15 - Mean % positively stained oospores for each treatment in the 1st trial testing heating of oospores in soil. Error bars show standard errors.

In Round 2 of this trial, heat treatment at 60°C for 4 hours and at 70°C for both 1 hour and 4 hours achieved similar reductions in percent positive stain (Table 3, Fig. 16). However exposure for 1 hour at 60°C achieved no significant reduction. Results were similar for both dry and wet soil. Heat treatment of exposed mesh achieved much less reduction than comparable treatment of mesh buried in soil.

Table 3 - Mean % positively stained oospores for each treatment in the 2nd trial testing heating of oospores in soil.

Exposure method	Temperature (°C)	Exposure time (hours)	% positive stain	
Mesh	room temp	0	85	a
	60	4	83	a
	70	1	77	ab
	70	4	60	b
Dry soil	60	1	72	ab
	60	4	6	c
	70	1	14	c
	70	4	3	c
Wet soil	60	1	87	a
	60	4	3	c
	70	1	3	c
	70	4	4	c

Values followed by the same letter in the final column do not differ significantly (p=0.05

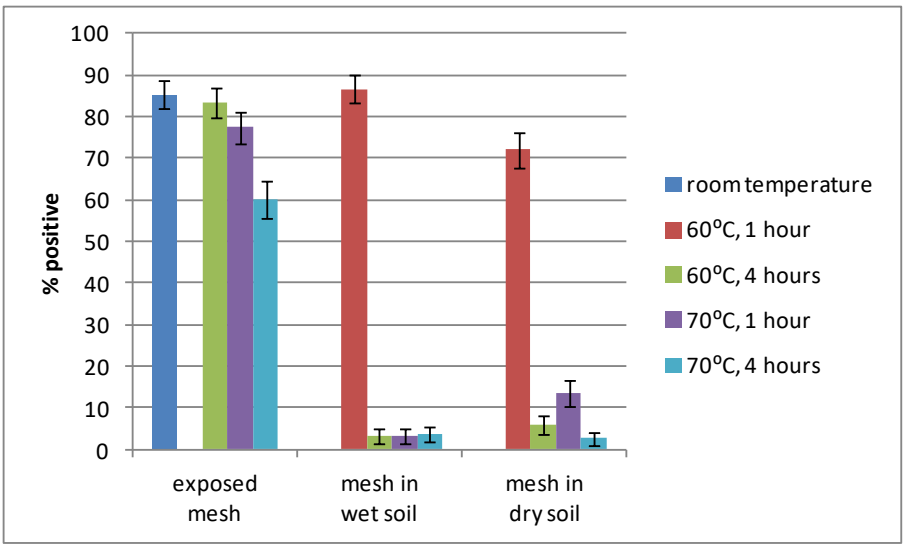

Fig. 16 - Mean % positively stained oospores for each treatment in the 2nd trial testing heating of oospores in soil. Error bars show standard errors.

Exposing the oospores (embedded on mesh attached to pieces of log) to dry heat gave a markedly different result to that achieved when the oospores were buried in soil. At 60°C for four hours there was no difference from the room temperature control (ca. 22°C) in contrast to both the wet and dry soils where the respective mean viability was 3% and 6%. For oospores in the soil the 70°C treatments, whether for 1 or 4 hours, were not significantly more effective than the 60°C for 4 hours. One hour at 60°C was not effective; results were not significantly different from the room temperature of exposed mesh controls.

General discussion

Neither Trigene solutions (2%, 5% and 10%), salt water immersion for short periods of time nor fumigation with metam sodium achieved significant deactivation of PTA oospores. Whereas the Trigene and salt water immersion results were not unexpected the failure of metam sodium fumigation was a surprise. In fact the treatment appeared to enhance the viability in contrast to the untreated soil control. Further examination of this result may be warranted.

The lowest pH level tested reduced viability of oospores to 10-20% regardless of exposure time. Complete deactivation occurred only at pH levels 9 and 10 with exposure for 48 hours.

Exposure to temperatures of 55°C and above (60°C and 70°C) in solution was effective at reducing viability to extremely low levels. For many of the temperature/time combinations tested there were no positively stained spores (Appendix A: 5-7) in some of the replicates. However the very occasional finding of a stained oospore meant that mean treatment results were seldom zero. It is possible that these occasional stained spores were more of an artefact than a reality.

Exposing the oospores embedded on mesh attached to pieces of log to dry heat gave a markedly different result to that achieved when the oospores were buried in soil. These results suggest that heat alone may be just one factor contributing to the deactivation observed. Potentially the moisture stimulates the initiation of germination and the spores thereby become vulnerable. It is possible that some chemical or enzymic activity within soil also has an influence in this stimulation. El Hamalawi & Erwin (1986) found that a short immersion of oospores of *P. megasperma* f.sp. *medicaginis* in 0.05% KMnO4 markedly increased germination. Working with the same species Salvatore et al. (1973) and Banihashemi & Mitchell (1976) found germination of *P. megasperma* f.sp. *medicaginis* and *P. cactorum* respectively was enhanced by application of snail-gut enzyme.

Differences between isolates used in some tests were observed. The isolates did not consistently rank in the same order in the tests suggesting that differences in the age of the oospore preparation may be important. Although these age differences were not great we do not have sufficient knowledge of the behaviour of this species of *Phytophthora* to determine how relevant this feature might be. Isolate differences were seldom significant in a single test.

The application of heat is likely to be the most effective treatment for ensuring the complete deactivation of oospores. The desired temperature could be achieved in different ways. Soil solarisation has been employed in the field to reduce levels of many pathogens including *Phytophthora* spp. Composting has been used for collected material. Steam has been applied to gravel and waste collected from contaminated sites.

Composting of plant waste infected with *Phytophthora* spp. has been examined as a potential management option (Noble et al. 2011) as temperatures reached in well-managed composting systems are usually high enough to eradicate plant pathogens. Eradication in this research was defined as 'reducing the level to below detectable limits'.

The effects of temperature–time combinations during composting on 64 plant pathogens (fungi, plasmodiophoromycetes, oomycetes, bacteria, viruses and nematodes were included) were reviewed by Noble & Roberts (2004). For all but five pathogens a peak temperature of 64–70°C and duration of 21 days, were sufficient to reduce numbers to below the detection limits of the tests used. Noble & Roberts (2004) commented that shorter periods and/or lower temperatures than those quoted in these tests may be satisfactory for eradication, but this had not always been examined in detail in composting systems. The eradication of pathogens from organic material is not solely a result of the heating process but also due to the production of toxic compounds, the lytic activity of enzymes formed in the compost and to microbial antagonism. Coventry et al. (2002) suggested that some of the compounds produced in the early stages of the compost process would stimulate the resting stages of fungi into germination and these would therefore become more susceptible to elimination. Notwithstanding the contribution of these processes it is the heat generated during the thermophilic high temperature phase of aerobic composting that is the most important factor in the death of micro-organisms (Bollen & Volker, 1996).

However successful composting is dependent on a high component of plant tissue to reach the desired temperatures and is therefore not likely to be a good option for samples with a high proportion of inorganic material such as those under consideration for soil contaminated with PTA oospores. It does appear to be a viable option for contaminated plant tissue.

Another method of applying heat to soil samples to reduce or eradicate targeted microorganisms is by soil solarisation. It involves laying clear plastic sheeting over cultivated, moist soil for four to six weeks in summer when air temperatures are high. The soil temperatures rise to well above 40°C and may be as high as 60ºC

(varying with air temperature and soil type) (Katan 1981). The process has been demonstrated to kill a range of pathogens including *Phytophthora* (Juarez-Palacios et al. 1991; Kaewruang et al. 1989; Wicks 1988).

Katan (1981) reviewed the extensive body of work on solar soil heating carried out to date. In a series of trials carried out in Israel temperatures of covered soil at 5 and 20 cm deep were 45-55 ºC and 39-45 ºC when average daily temperatures were 25-26 ºC. Juarez-Palacios et al. (1991) examined the effect of solarisation on three species of *Phytophthora* including one high-temperature isolate of the oospore-producing species *P. megasperma*. In field trials nylon bags containing inoculum was buried in soil to depths up to 45 cm for up to four weeks and isolate survival was determined by baiting flooded soil samples. In this trial temperatures reached a maximum temperature of 45°C at 15 cm depth and 33°C at 45 cm depth. Although most of the *P. megasperma* was eliminated (85-98% colonisation of baits at 15 cm depth) there was a residual survival which may have been in part due to the existence of oospores.

Soil solarisation, as a disinfestation method, has advantages in that it is relatively cheap to employ and is not hazardous to the user. It can however only be used in areas where the air temperatures are high enough and is generally applicable only in the summer months.

Commercial steam applicators are available in a variety of sizes (e.g. Weedtechnics 2013 (viewed online March)). To determine specifications for treatments of collected soil/debris using a steam applicator trials would need to carried out.

Researchers examining the survival of *Phytophthora* oospores in soil, and in other media have often used either direct plating of infested material or bioassay using a variety of baiting techniques to determine whether there is viable inoculum remaining. Typically the temperature/time combinations that we used in the experiments discussed here would be sufficient to achieve a nil recovery using the detection methods employed. However we frequently found a very low percentage of oospores that stained positively. As this percentage remained the same (not statistically different) with temperatures increasing from 55-70 ºC it is likely that this result may be an artefact of the tetrazolium bromide staining method. To clarify this result a repeat of selected treatments with both oospore staining and a bioassay for viability could be employed. Treated soil could also be held for longer periods of time than we used, and in optimal conditions for germination, before viability testing (by any method). Dispersal of oospores through the soil rather than burying pieces of mesh with embedded oospores would provide an opportunity to test sub-samples at different intervals following treatment (not applicable to tetrazolium bromide staining but appropriate for bioassay) and determine whether germination and subsequent soil colonisation had occurred.

Conclusions

For practical purposes the application of heat is likely to be the most effective and practical treatment for deactivation of PTA oospores in samples of contaminated soil collected from equipment or footwear. Results of this work indicate that temperatures of 60 - 70°C applied to wet soil or through a steam applicator for periods of 4 hours would result in total kill. A safety margin could be applied. At temperatures higher than 70°C shorter treatment periods are likely to be effective.

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Appendix A

1. Data - Trigene experiments

Treatment		isolate	Time (mins)	Rep.	No. stained oospores
Control	deionised water	3687	30	1	64
Control	deionised water	3687	30	2	57
Control	deionised water	3687	30	3	71
Trigene 2%		3687	30	1	68
Trigene 2%		3687	30	2	69
Trigene 2%		3687	30	3	76
Trigene 5%		3687	30	1	56
Trigene 5%		3687	30	2	62
Trigene 5%		3687	30	3	75
Trigene 10%		3687	30	1	31
Trigene 10%		3687	30	2	39
Trigene 10%		3687	30	3	62
Control	deionised water	3687	120	1	72
Control	deionised water	3687	120	2	76
Control	deionised water	3687	120	3	69
Trigene 2%		3687	120	1	65
Trigene 2%		3687	120	2	62
Trigene 2%		3687	120	3	61
Trigene 5%		3687	120	1	80
Trigene 5%		3687	120	2	71
Trigene 5%		3687	120	3	75
Trigene 10%		3687	120	1	72
Trigene 10%		3687	120	2	84
Trigene 10%		3687	120	3	65
Control	deionised water	3709	30	1	88
Control	deionised water	3709	30	2	91
Control	deionised water	3709	30	3	95
Trigene 2%		3709	30	1	95
Trigene 2%		3709	30	2	93
Trigene 2%		3709	30	3	91
Trigene 5%		3709	30	1	90
Trigene 5%		3709	30	2	91
Trigene 5%		3709	30	3	95
Trigene 10%		3709	30	1	88
Trigene 10%		3709	30	2	87
Trigene 10%		3709	30	3	93
Control	deionised water	3709	120	1	82
Control	deionised water	3709	120	2	75
Control	deionised water	3709	120	3	77
Trigene 2%		3709	120	1	82
Trigene 2%		3709	120	2	94
Trigene 2%		3709	120	3	86
Trigene 5%		3709	120	1	84

Trigene 5%	3709	120	2	90
Trigene 5%	3709	120	3	93
Trigene 10%	3709	120	1	75
Trigene 10%	3709	120	2	83
Trigene 10%	3709	120	3	93

2. Data - Salt water experiments

Treatment		isolate	Time (mins)	Rep.	No. stained oospores
Control	deionised water	3709	1	1	89
Control	deionised water	3709	1	2	88
Control	deionised water	3709	1	3	93
Saltwater 3.5%		3709	1	1	88
Saltwater 3.5%		3709	1	2	92
Saltwater 3.5%		3709	1	3	89
Saltwater 3.8%		3709	1	1	96
Saltwater 3.8%		3709	1	2	91
Saltwater 3.8%		3709	1	3	94
Control	deionised water	3709	15	1	92
Control	deionised water	3709	15	2	89
Control	deionised water	3709	15	3	89
Saltwater 3.5%		3709	15	1	88
Saltwater 3.5%		3709	15	2	88
Saltwater 3.5%		3709	15	3	87
Saltwater 3.8%		3709	15	1	91
Saltwater 3.8%		3709	15	2	89
Saltwater 3.8%		3709	15	3	88
Control	deionised water	3681	1	1	95
Control	deionised water	3681	1	2	93
Control	deionised water	3681	1	3	91
Saltwater 3.5%		3681	1	1	90
Saltwater 3.5%		3681	1	2	86
Saltwater 3.5%		3681	1	3	82
Saltwater 3.8%		3681	1	1	91
Saltwater 3.8%		3681	1	2	85
Saltwater 3.8%		3681	1	3	83
Control	deionised water	3681	15	1	94
Control	deionised water	3681	15	2	94
Control	deionised water	3681	15	3	95
Saltwater 3.5%		3681	15	1	90
Saltwater 3.5%		3681	15	2	86
Saltwater 3.5%		3681	15	3	82
Saltwater 3.8%		3681	15	1	91
Saltwater 3.8%		3681	15	2	91
Saltwater 3.8%		3681	15	3	82

3. Data - Fumigation experiments

Treatment		Rep.	No. stained oospores
lab control		1	86
lab control		2	79
lab control		3	75
sand	untreated	1	49
sand	untreated	2	75
sand	untreated	3	72
sand	0.05 ml	1	84
sand	0.05 ml	2	82
sand	0.05 ml	3	84
sand	0.25 ml	1	93
sand	0.25 ml	2	87
sand	0.25 ml	3	88
sand	0.5 ml	1	88
sand	0.5 ml	2	83
sand	0.5 ml	3	88
soil	untreated	1	59
soil	untreated	2	67
soil	untreated	3	77
soil	0.05 ml	1	87
soil	0.05 ml	2	76
soil	0.05 ml	3	80
soil	0.25 ml	1	88
soil	0.25 ml	2	64
soil	0.25 ml	3	84
soil	0.5 ml	1	82
soil	0.5 ml	2	84
soil	0.5 ml	3	75

4. Data - pH experiments

Treatment (pH)	Time (hours)	Rep.	No. stained oospores
3	2	1	12
3	2	2	9
3	2	3	15
3	4	1	14
3	4	2	11
3	4	3	17
3	12	1	10
3	12	2	15
3	12	3	18
3	24	1	14
3	24	2	16
3	24	3	11
3	48	1	12
3	48	2	18
3	48	3	17
4	2	1	21
4	2	2	27
4	2	3	30
4	4	1	31
4	4	2	34
4	4	3	28
4	12	1	30
4	12	2	18
4	12	3	19
4	24	1	9
4	24	2	19
4	24	3	22
4	48	1	26
4	48	2	20
4	48	3	11
5	2	1	36
5	2	2	27
5	2	3	15
5	4	1	12
5	4	2	27
5	4	3	21
5	12	1	27
5	12	2	45
5	12	3	45
5	24	1	12
5	24	2	6
5	24	3	6
5	48	1	3
5	48	2	6
5	48	3	3
6	2	1	66

6	2	2	72
6	2	3	59
6	4	1	15
6	4	2	18
6	4	3	31
6	12	1	27
6	12	2	30
6	12	3	9
6	24	1	27
6	24	2	24
6	24	3	21
6	48	1	21
6	48	2	15
6	48	3	18
7	2	1	50
7	2	2	54
7	2	3	57
7	4	1	53
7	4	2	39
7	4	3	41
7	12	1	38
7	12	2	28
7	12	3	40
7	24	1	27
7	24	2	21
7	24	3	34
7	48	1	20
7	48	2	18
7	48	3	16
8	2	1	55
8	2	2	61
8	2	3	48
8	4	1	39
8	4	2	26
8	4	3	38
8	12	1	24
8	12	2	41
8	12	3	28
8	24	1	17
8	24	2	13
8	24	3	30
8	48	1	12
8	48	2	15
8	48	3	11
9	2	1	64
9	2	2	62
9	2	3	48
9	4	1	34
9	4	2	36
9	4	3	28
9	12	1	20

9	12	2	44
9	12	3	16
9	24	1	6
9	24	2	8
9	24	3	8
9	48	1	0
9	48	2	0
9	48	3	0
10	2	1	69
10	2	2	50
10	2	3	40
10	4	1	43
10	4	2	40
10	4	3	43
10	12	1	25
10	12	2	33
10	12	3	32
10	24	1	6
10	24	2	10
10	24	3	9
10	48	1	0
10	48	2	0
10	48	3	0

5. Data - Heated oospore solutions

Treatment (temperature)	Isolate	Time (hr)	No. stained oospores
RT control	3687	24	24
RT control	3687	24	12
RT control	3687	24	15
55 C	3687	4	0
55 C	3687	4	0
55 C	3687	4	0
55 C	3687	6	1
55 C	3687	6	0
55 C	3687	6	3
55 C	3687	12	0
55 C	3687	12	0
55 C	3687	12	0
55 C	3687	24	0
55 C	3687	24	0
55 C	3687	24	2
RT control	3709	24	57
RT control	3709	24	58
RT control	3709	24	65
55 C	3709	4	0
55 C	3709	4	0
55 C	3709	4	1
55 C	3709	6	1
55 C	3709	6	0
55 C	3709	6	0
55 C	3709	12	0
55 C	3709	12	0
55 C	3709	12	0
55 C	3709	24	0
55 C	3709	24	0
55 C	3709	24	0
RT control	3681	24	65
RT control	3681	24	67
RT control	3681	24	59
55 C	3681	6	23
55 C	3681	6	5
55 C	3681	6	4
55 C	3681	24	3
55 C	3681	24	4
55 C	3681	24	6
RT control	3681	24	88
RT control	3681	24	76
RT control	3681	24	72
60 C	3681	4	2
60 C	3681	4	1
60 C	3681	4	0
60 C	3681	6	0

60 C	3681	6	0
60 C	3681	6	0
60 C	3681	9	0
60 C	3681	9	1
60 C	3681	9	1
60 C	3681	19	0
60 C	3681	19	0
60 C	3681	19	0
RT control	3709	24	80
RT control	3709	24	73
RT control	3709	24	71
60 C	3709	4	10
60 C	3709	4	2
60 C	3709	4	15
60 C	3709	6	9
60 C	3709	6	2
60 C	3709	6	8
60 C	3709	9	16
60 C	3709	9	7
60 C	3709	9	7
60 C	3709	19	0
60 C	3709	19	0
60 C	3709	19	1
RT control	3681	2	23
RT control	3681	2	25
RT control	3681	2	23
70 C	3681	0.5	1
70 C	3681	0.5	4
70 C	3681	0.5	2
70 C	3681	1	0
70 C	3681	1	1
70 C	3681	1	2
70 C	3681	2	1
70 C	3681	2	1
70 C	3681	2	3
RT control	3709	2	54
RT control	3709	2	57
RT control	3709	2	65
70 C	3709	0.5	1
70 C	3709	0.5	11
70 C	3709	0.5	8
70 C	3709	1	4
70 C	3709	1	3
70 C	3709	1	2
70 C	3709	2	1
70 C	3709	2	0
70 C	3709	2	1
RT control	3687	2	80
RT control	3687	2	65
RT control	3687	2	69

70 C	3687	0.5	5
70 C	3687	0.5	0
70 C	3687	0.5	8
70 C	3687	1	6
70 C	3687	1	0
70 C	3687	1	1
70 C	3687	2	0
70 C	3687	2	1
70 C	3687	2	0

6. Data - Heated oospores in soil - Trial 1

Treatment (temperature)	Time (hrs)	Soil	Isolate	No. stained oospores
50	4	dry	3681	5
50	4	dry	3681	4
50	4	dry	3681	8
50	4	dry lid off	3681	10
50	4	dry lid off	3681	18
50	4	dry lid off	3681	2
50	4	steam	3681	4
50	4	steam	3681	2
50	4	steam	3681	5
50	24	dry	3687	18
50	24	dry	3681	0
50	24	dry	3681	3
50	24	dry lid off	3687	7
50	24	dry lid off	3681	0
50	24	dry lid off	3681	0
50	24	steam	3681	0
50	24	steam	3681	0
50	24	steam	3681	0
60	1	dry	3681	0
60	1	dry	3681	1
60	1	dry	3681	4
60	1	dry lid off	3681	0
60	1	dry lid off	3681	1
60	1	dry lid off	3681	0
60	1	steam	3681	6
60	1	steam	3681	1
60	1	steam	3681	1
60	4	dry	3687	2
60	4	dry	3681	1
60	4	dry	3681	5
60	4	dry lid off	3687	72
60	4	dry lid off	3681	73
60	4	dry lid off	3681	44
60	4	steam	3687	0
60	4	steam	3681	1
60	4	steam	3681	0
60	24	dry	3687	9
60	24	dry	3681	20
60	24	dry	3681	41
60	24	dry lid off	3687	1
60	24	dry lid off	3681	5
60	24	dry lid off	3681	2
60	24	steam	3687	0

60	24	steam	3681	2
60	24	steam	3681	3
70	1	dry	3687	12
70	1	dry	3681	4
70	1	dry	3681	7
70	1	dry lid off	3687	72
70	1	dry lid off	3681	8
70	1	dry lid off	3681	2
70	1	steam	3687	13
70	1	steam	3681	9
70	1	steam	3681	9
70	4	dry	3687	0
70	4	dry	3681	5
70	4	dry	3681	5
70	4	dry lid off	3687	
70	4	dry lid off	3681	
70	4	dry lid off	3681	0
70	4	steam	3687	2
70	4	steam	3681	0
70	4	steam	3681	0
70	24	dry	3687	4
70	24	dry	3681	0
70	24	dry	3681	0
70	24	dry lid off	3687	3
70	24	dry lid off	3681	1
70	24	steam	3687	8
70	24	steam	3681	5
70	24	steam	3681	8
RT - control	mesh untreated		3681	41
RT - control	mesh untreated		3687	45
RT - control	mesh untreated		3687	35
RT - control	mesh untreated		3681	42
RT - control	mesh untreated		3681	53
RT - control	mesh untreated		3681	64
RT - control	mesh untreated		3681	78
RT - control	mesh untreated		3681	48
RT - control	mesh in soil		3681	82
RT - control	mesh in soil		3681	66
RT - control	mesh in soil		3681	60

7. Data - Heated oospores in soil - Trial 2

Temperature	Moisture	Time (hours)	No. stained oospores
RT	nil		83
RT	nil		84
RT	nil		89
60 C	exposed	4	77
60 C	exposed	4	85
60 C	exposed	4	88
60 C	dry soil	1	57
60 C	dry soil	1	78
60 C	dry soil	1	81
60 C	dry soil	4	8
60 C	dry soil	4	3
60 C	dry soil	4	7
60 C	wet soil	1	84
60 C	wet soil	1	89
60 C	wet soil	1	87
60 C	wet soil	4	3
60 C	wet soil	4	4
60 C	wet soil	4	3
70 C	exposed	1	62
70C	exposed	1	88
70 C	exposed	1	82
70C	exposed	4	58
71 C	exposed	4	58
70C	exposed	4	64
70C	dry soil	1	15
70 C	dry soil	1	9
70C	dry soil	1	17
70 C	dry soil	5	1
70C	dry soil	5	2
70 C	dry soil	5	5
70C	wet soil	1	1
70 C	wet soil	1	4
70C	wet soil	1	5
70 C	wet soil	5	7
70C	wet soil	5	2
70 C	wet soil	5	2